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Cytogeography of *Caltha palustris* (Ranunculaceae) from China

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Abstract: Twenty-three *C. palustris* accessions and ten *C. scaposa* accessions have been cytologically investigated using the traditional chromosome tableting technique and flow cytometry (FCM), in order to investigate the evolution of *C. palustris* and *C. scaposa* in *Caltha* of Ranunculaceae. *Caltha palustris* was found to be a polyploid complex, which contained tetraploids ($2n = 4x = 32$), hexaploids ($2n = 6x = 48$), and octoploids ($2n = 8x = 64$), and *C. scaposa* were tetraploids ($2n = 4x = 32$) and octoploids ($2n = 8x = 64$). Tetraploids were common in *C. palustris* and *C. scaposa*; however, hardly any diploids were discovered. This finding may be explained by cytotype adaptive differences to the underlying heterogeneity of environmental factors. Most accessions of *C. palustris* and *C. scaposa* were from extreme habitats, such as the alpine mountains in the Qinghai–Tibetan Plateau. Ancestral diploids may have existed in this region during glacial periods and colonized most regions at the end of the glaciation cycles. However, individuals with other ploidy levels may gradually replace diploids, because of their increased fitness in changing environment. Moreover, there were two possible evolutionary colonization routes: one from Gan'su to Yunnan, and the other from Tibet to Yunnan. Molecular phylogeny have shown that *C. scaposa* is closely related to *C. palustris*, the chromosome size of *C. scaposa* was smaller than that of *C. palustris*, *C. scaposa* may be a relatively derived evolutionary taxon. More samples need to be analyzed in the future to better elucidate *C. scaposa* cytogeography because of only 10 accessions.
Key words: cytogeography, *C. palustris*, *C. scaposa*, polyploidy

国产驴蹄草的细胞地理学研究

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摘要：为探讨国产毛茛科（Ranunculaceae）驴蹄草属（*Caltha*）两种植物的演化，该文利用传统染色体压片技术和流式细胞术，并结合前人染色体研究结果，对我国驴蹄草 23 个居群和花葶驴蹄草 10 个居群进行了细胞学研究。结果表明：驴蹄草是由四倍体（$2n = 4x = 32$）、

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六倍体（$2n = 6x = 48$）和八倍体（$2n = 8x = 64$）构成的多倍体复合群，花葶驴蹄草具有四倍体（$2n = 4x = 32$）和八倍体（$2n = 8x = 64$）两种倍性水平。驴蹄草和花葶驴蹄草均四倍体较为常见，目前尚未有二倍体报道。由于驴蹄草和花葶驴蹄草大部分居群采自青藏高原地区，可能在冰期时存在古二倍体，其适应性较弱，逐渐被其它的倍性取代，是由于不同细胞型对环境适应性的结果。驴蹄草可能存在两条进化路线：一条是从甘肃到达云南，另一条是从西藏到达云南。前期分子系统学研究显示花葶驴蹄草与驴蹄草亲缘关系较近，本研究结果中花葶驴蹄草染色体比驴蹄草要小，花葶驴蹄草可能比驴蹄草相对进化。目前花葶驴蹄草只有 10 个居群，还需进一步增加居群量来解析其演化路线。
关键词：细胞地理，驴蹄草，花葶驴蹄草，多倍化

Polyploidy, the duplication of entire sets of chromosomes, is a key process in the evolution and diversification of vascular plants (Hegarty et al., 2013; Otto & Whitton, 2000). Previous studies found that polyploids are better able to adapt to stress or novel niches than their diploid progenitors (Ehrendorfer, 1980; Grant, 1981; Levin, 2004; Morton, 1993; Otto & Whitton, 2000; Stebbins, 1985). Furthermore, intraspecific variation in ploidy level is frequently observed in angiosperms (Kolář et al., 2015; Wood et al., 2009). It is known that polyploidization is one of the few speciation processes that may operate in sympatry, due to the possible immediate emergence of reproductive isolation between individuals with different ploidy levels (Husband & Sabara, 2003). Therefore, the geographic distribution of cytotypes could provide valuable information about the origin and maintenance of different ploidy levels (Baack, 2004; Kolář et al., 2009; Rieseberg & Willis, 2007; Segraves et al., 1999).

The perennial herb *Caltha palustris* grows from 600–4 000 m in mountain regions, valleys, marshlands, forests, streams, and on grassy slopes in the north temperate region (Wang et al., 2001). After *C. palustris* was first described by Linnaeus (1753), great variability of some morphological characters was described in this species, such as plant size, leaf shape and size, leaf margins, flowers, mature follicles, rooting at nodes, tepal number and color, and seed color and symmetry (Smit, 1973; Kumar & Singhal, 2008). It was previously shown that morphological diversity is a product of environmental conditions (Blagojevic et al., 2013). The current study primarily focused on cytotype distribution in the *C. palustris* complex, which includes tetraploids (Wang et al., 2013; Yang, 2002; Yuan & Yang, 2006), hexaploids (Parfenov & Dmitrieva, 1985; Wang et al., 2013; Yang, 2002; Yuan & Yang, 2006), and octoploids (Wang et al., 2013; Yang, 2002; Yuan & Yang, 2006) ($x = 8$, Langlet, 1927). Furthermore, molecular phylogenetic evidence also showed that *C. scaposa* is sister to *C. palustris* (with 100% bootstrap support) (Cheng & Xie, 2014; Schuettpelz & Hoot, 2004). *Caltha scaposa* is endemic to Sino-Himalaya, and grows from 2800 to 4100 m in wet parts of alpine meadows and valleys. Only two cytotypes have been detected: tetraploids (Wang et al., 2013) and octoploids (Wang et al., 2013; Yuan & Yang, 2006) ($x = 8$, Langlet, 1927). The existence of different cytotypes in *C. palustris* and *C. scaposa* may indicate strong spatial segregation. As a result of inche differentiation (Ehrendorfer, 1980; Lewis, 1980), reproductive exclusion (Levin, 1975; Van-Dijk & Bakx-Schotman, 1997), and historical factors (Ančev, 2006), these distinct cytotypes may experience differential reproductive success and occurrence of particular evolutionary constraints or demographic stochasticity (Munoz-Pajares et al., 2017).

By conducting a novel analysis of previous cytotype distribution data, we herein present a cytogeographical study of *C. palustris* and *C. scaposa* in China. Our aims in this study were to: 1) assess the geographic distribution of different cytotypes in *C. palustris* and *C. scaposa* to propose a scenario of dispersal events; and 2) determine the major driving force of speciation in *C. palustris* and *C. scaposa*.

1 Materials and Methods

1.1 Taxon sampling

In this study, we sampled six *C. palustris* accessions and four *C. scaposa* accessions (Table 1). In total, 15–20 plants from each population were sampled. Geographical coordinates were recorded in the field using a GPS instrument. Living plants were cultivated in a greenhouse, and voucher specimens were deposited in the herbarium at Kunming Institute of Botany, Chinese

Academy of Sciences. We performed cytogeographical analysis using these accessions and previously reported data (Yang, 2002; Yuan & Yang, 2006; Table 2).

1.2 Chromosome number

Root tips were collected from each individual and pretreated with a solution of 0.002 mol·L^{-1} 8-hydroxyquinoline at 20–21 °C for 4–5 h. After fixation for 50 min in Carnoy's solution (3:1 ethanol : acetic acid) at 4 °C, the root tips were dissociated in a mixture of 1 N HCl and 45% acetic acid (1:1) at 60 °C for 30 s, stained with 1% acetic orcein for 2–3 h and squashed on a glass slide (Wang et al., 2013). Chromosome numbers were determined for each accession from at least 50 cells of at least two seedlings by mitotic observations. Mitotic interphase nuclei and prophase chromosomes preparations followed Tanaka (1971, 1977, 1987), and the designation of the centromeric position followed Levan et al. (1964). Karyotype asymmetry was classified according to Stebbins (1971).

1.3 Flow cytometry and DNA ploidy level determination

Propidium iodide flow cytometry (FCM) analysis was performed using fresh leaf samples from our greenhouse. Approximately 0.5 cm^2 of leaf material was finely diced using a new razor blade in a Petri dish that contained 1,500–2,000 µL of WPB nuclear solution buffer (0.2 mol·L^{-1} Tris · HCl, 4 mmol·L^{-1} MgCl · 6H_2O, 2 mmol·L^{-1} EDTA Na_2 · 2H_2O, 86 mmol·L^{-1} NaCl, 10 mmol·L^{-1} $Na_2S_2O_5$, 1% PVP-10, 1% [v/v] Triton X-100, pH 7.5) (Tian et al., 2011). The nuclear suspension was then filtered through disposable filters (30 µm) to remove cell debris, and stained with 150 µL propidium iodide (50 µg·mL^{-1}; including RNAse [500 µg·mL^{-1}]) for 10 min. Samples were analyzed on a CyFlow Space (Partec, Münster, Germany) flow cytometer equipped with a blue laser operating at 488 nm. At least 5000 nuclei were measured for each sample. FlowMax ver. 2.82 was used to analyze the resulting histograms. By comparison with a known ploidy level (4*x*; yyp04), we estimated the ploidy levels of other samples based on the histograms. The ploidy level of each sample was calculated as described by Tian et al., 2011:

Ploidy level of sample = (mean of sample peak/mean of standard peak) ∗ ploidy level of the standard species

2 Results

2.1 Chromosome counts and DNA ploidy level determination

In this study, ploidy levels included 13 4*x* *C. palustris*, one 6*x* *C. palustris*, nine 8*x* *C. palustris*, seven 4*x* *C. scaposa*, and three 8*x* *C. scaposa* accessions (Table 2); these specimens were collected from Gan'su (one accession), Yunnan (16 accessions), Sichuan (four accessions), Tibet (one accession), Guizhou (one accession), and Qinghai (one accession). Metaphase chromosomes of eight accessions are shown in Fig. 1. We successfully estimated the ploidy levels of two *C. palustris* accessions (yyp09 and yyp10) by FCM at 4*x* and 8*x* (Fig. 2).

2.2 Cytogeography

The ploidy distribution of *C. palustris* and *C. scaposa* was revealed based on currently available data (Fig. 3). All *C. palustris* accessions were single-ploidy, although our sample was very limited in some accessions; however, secondary constriction chromosomes were observed in three *C. palustris* accessions (Table 2). The tetraploid cytotype was more common than the other cytotypes (hexaploids and octoploids). Moreover, the tetraploid karyotype also exhibited obvious variation among accessions. The samples from Diqing State (Yunnan) included tetraploid, hexaploid, and octoploid cytotypes. Two cytotypes (tetraploids and octoploids) were found in Lijiang (Yunnan). Only one cytotype existed in Tewo (Gansu), Dali (Yunnan), Gongshan (Yunnan), Hongyuan (Sichuan), Nayong (Guizhou), and Zuogong (Tibet). All *C. scaposa* accessions were single-ploidy, and the tetraploid cytotype was also common. Two cytotypes (tetraploids and octoploids) existed in Sichuan, and one cytotype each in Tibet, Qinghai, and Yunnan. In addition, only one contact areas between different cytotype were detected (Fig. 3). In the Xiaozhongdian accession, a region of overlap between the ranges of 4*x* *C. palustris* and 8*x* *C. scaposa* was observed.

3 Discussion

FCM offers a rapid and precise method for identifying taxa of different ploidy levels, enabling researchers to map the fine-scale distribution of ploidies within individual populations (Suda et al., 2004). FCM has been used in ploidy analysis, e.g., in *Ranunculus* (Ranunculaceae) (Cires et al., 2010) and *C. leptosepala* s.l. (Wefferling et al., 2017). In our study, ploidy levels of two accessions (yyp09 and yyp10) were estimated by FCM. The current study revealed that *C. palustris* may be viewed as a polyploid complex, which presents clear patterns of cytotype

distribution. Polyploidy is a prevalent phenomenon in the chromosomal evolution of extant species and genera (Otto & Whitton, 2000), and it may have contributed to the origin of flowering plants (De Bodt et al., 2005). As a result, plant scientists have recognized that polyploid lineages may have complex relationships with each other and their diploid ancestors, making application of species concepts problematic (Soltis et al., 2007, 2009).

The *C. palustris* polyploid complex showed a varied cytotype distribution. No diploids and few hexaploids were found in this study, but tetraploid and octoploid cytotypes were common and widespread. Similarly, in *C. scaposa*, tetraploids and octoploids were common, whereas diploids and hexaploids were not found. Such distribution patterns are often explained by cytotype adaptive differences to the underlying heterogeneity of environmental factors (Lewis, 1980). All accessions except for the Guizhou and Gan'su accessions were from extreme habitats, such as alpine mountains in the Qinghai–Tibetan Plateau. Polyploidy is common in plants from cold climates with harsh and stressful environments (Grant, 1981; Löve & Löve, 1949, 1967). Therefore, a relatively high frequency of polyploidy was observed in this species. Ancestral diploids may have been present in this region during glacial periods and colonized most regions at the end of the glaciation cycles. However, other ploidy levels may gradually replace diploids, because of their increased fitness in changing environment (Cui et al., 2008).

The chromosome counts observed in the *C. palustris* complex indicate that ploidy changes may have been important in its evolution. Chromosome counts often show obvious differences in different accessions within a particular species. Our analysis showed that the Hengduan Mountains may be better viewed as a polyploid complex of diploids, tetraploids, and hexaploids. Symmetrical karyotypes are widely accepted to be more primitive than asymmetrical ones (Stebbins, 1971). In our combined data (Table 1), the accessions from Zhongdian (Yunnan) with different types (3B, 3C), AI (11.39, 7.34, 5.96), and secondary constrictions showed the highest asymmetric tendencies. Therefore, we speculate that two possible evolutionary trends may exist: one from Gan'su to Yunnan, and another from Tibet to Yunnan.

Molecular phylogeny have shown that *C. scaposa* is closely related to *C. palustris* (Cheng & Xie, 2014; Schuettpelz & Hoot, 2004). *Caltha scaposa* cytotype distribution was determined from only 10 populations; therefore, *C. scaposa* cytogeography could not be comprehensively analyzed. Moreover, the chromosome size of this species was smaller than that of *C. palustris*. The size of the chromosome is also a feature subject to evolutionary change, the direction of chromosome evolution could be toward a decrease in chromosome size (Martel et al., 2004). Therefore, smaller chromosomes may be a relatively derived evolutionary character. Consequently, in the future, additional samples need to be analyzed to better elucidate *C. scaposa* cytogeography.

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表 1 驴蹄草和花葶驴蹄草凭证标本信息

Table 1 Voucher information of *Caltha palustris* and *C. scaposa* in this study

Taxon	Locality	Voucher	Latitude	Longitude	Altitude
Caltha palustris	Xiaozhongdian Town, Zhongdian Country, Yunnan	yyp04	27°33′11″	99°48′46″	3220
	Tieciling, Tewo, Gansu		35°44′6″	104°17′10″	3468
	Huadianba, Cangshan Country, Dali, Yunnan		25°38′39″	100°10′11″	1978
	Xiaohuadianba, Cangshan Country, Dali, Yunnan		25°38′39″	100°10′11″	1978
	Bitahai, Zhongdian Country, Yunnan		27°49′26″	99°59′23″	3541
	Napahai, Zhongdian Country, Yunnan		27°53′22″	99°39′13″	3282
	Pantiange, Weixi Country, Yunnan		27°19′50″	99°13′18″	2541
	Kangpu, Weixi Country, Yunnan		27°35′58″	99°0′56″	1724
	Ke’na, Weixi Country, Yunnan		27°10′37″	99°17′13″	2300
	Wenhai, Lijiang, Yunnan		26°58′26″	100°9′51″	3078
	Zhegu Mountain, Hongyuan, Sichuan		31°50′18″	102°39′48″	4073
	Ganba Village, Ju’ren Town, Nayong Country, Guizhou		28°20′22″	99°5′7″	1666
	Dongda Mountain, Zuogong Country, Tibet	yyp09	29°44′56″	97°57′53″	5156
	Tu’guan Village, Xiaozhongdian Town, Zhongdian Country, Yunnan		27°33′11″	99°48′46″	3220
	Tianbao Mountain, Zhongdian Country, Yunnan	yyp07	27°39′23″	99°55′3″	3080
	Bigutianchi, Zhongdian Town, Zhongdian Country, Yunnan	yyp08	27°33′11″	99°48′46″	3220
	Dulongjiang, Gongshan Country, Yunnan	yyp06	27°44′0″	98°21′0″	1431
	Wutoudi, Yulongxue Mountain, Lijiang, Yunnan		27°5′53″	100°10′30″	5427
	Hong Mountain, Zhongdian Country, Yunnan		27°11′3″	100°3′37″	3150
	Bigusang, Zhongdian Country, Yunnan		27°43′25″	99°41′25″	3339
	Yuhu, Yulongxue Mountain, Lijiang, Yunnan		27°5′53″	100°10′30″	5427
	Baimaxue Mountain, Deqin Country, Yunnan		28°20′22″	99°5′7″	4361
	LBaimaxue Mountain, Deqin Country, Yunnan	yyp10	28°20′22″	99°5′7″	4361
C. scaposa	Gao Mountain, Kangding Country, Sichuan	yyp02	29°59′54″	101°57′25″	2861
	Dege Country, Sichuan	yyp01	31°48′22″	98°34′51″	3290
	Daofu Country, Sichuan	yyp03	30°58′46″	101°7′30″	2979
	Shiqu Country, Sichuan		32°58′44″	98°6′10″	4178
	Hongyuan Country, Sichuan		32°47′27″	102°32′39″	3492
	Xindu Bridge, Kangding Country, Sichuan		30°2′33″	101°29′43″	2861
	Chengduo Country, Qinghai		33°22′9″	97°6′38″	3831
	A’ba Country, Sichuan	yyp05	31°53′57″	102°13′28″	2617
	Sejila Mountain, Linzhi Country, Tibet		29°56′36″	94°47′57″	3400
	Xiaozhongdian, Zhongdian, Yunnan		27°33′11″	99°48′46″	3220

表 2 驴蹄草和花葶驴蹄草细胞学特征

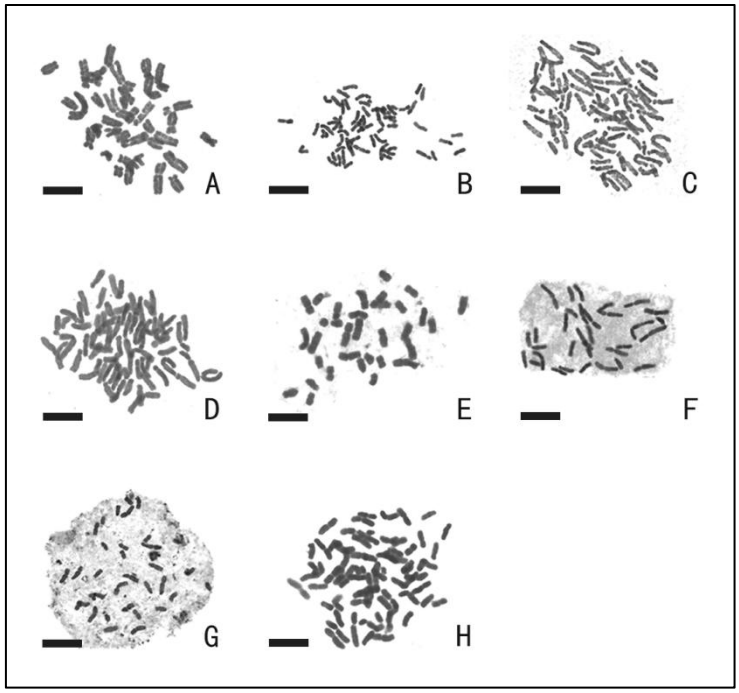
Table 2 Cytological characteristics of *Caltha palustris* and *C. scaposa* in this study

Taxon	Locality	Voucher	Ratio LC/SC	CL (μm) Mean ± SD	AI	Type	Karyotype formula	Reference
Caltha palustris	Xiaozhongdian Town, Zhongdian Country, Yunnan	yyp04	3.30	4.09 ± 1.19	7.34	2B	2*n*=4*x*=32=13m+13sm(1sec)+6st	
	Tieciling, Tewo, Gansu						2*n*=4*x*=32=20m+8sm+4st	Yang (2002), Yuan and Yang (2006)
	Huadianba, Cangshan Country, Dali, Yunnan						2*n*=4*x*=32=18m+6sm+8st	Yang (2002), Yuan and Yang (2006)
	Xiaohuadianba, Cangshan Country, Dali, Yunnan						2*n*=4*x*=32=18m+6sm+8st	Yang (2002), Yuan and Yang (2006)
	Bitahai, Zhongdian Country, Yunnan						2*n*=4*x*=32=18m+6sm+8st	Yuan and Yang (2006)
	Napahai, Zhongdian Country, Yunnan						2*n*=4*x*=32=8m+6sm+14st+4t	Yang (2002), Yuan and Yang (2006)
	Pantiange, Weixi Country, Yunnan						2*n*=4*x*=32=16m+8sm+8st	Yang (2002), Yuan and Yang (2006)
	Kangpu, Weixi Country, Yunnan						2*n*=4*x*=32=16m+8sm+8st	Yang (2002), Yuan and Yang (2006)
	Ke'na, Weixi Country, Yunnan						2*n*=4*x*=32=16m+8sm+8st	Yang (2002), Yuan and Yang (2006)
	Wenhai, Lijiang, Yunnan						2*n*=4*x*=32=6m+4sm+22st	Yang (2002), Yuan and Yang (2006)
	Zhegu Mountain, Hongyuan, Sichuan						2*n*=4*x*=32=18m+6sm+8st	Yuan and Yang (2006)
	Ganba Village, Ju'ren Town, Nayong Country, Guizhou						2*n*=4*x*=32=16m+2sm+14st	Wang et al. (2013)
	Dongda Mountain, Zuogong Country, Tibet	yyp09					4*x*	
	Tu'guan Village, Xiaozhongdian Town, Zhongdian Country, Yunnan						2*n*=6*x*=48=31m+11sm+6st	Yang (2002), Yuan and Yang (2006)
	Tianbao Mountain, Zhongdian Country, Yunnan	yyp07	4.07	4.74±1.74	11.39	3C	2*n*=8*x*=64=14m+28sm(1sec)+22st	
	Bigutianchi, Zhongdian Town, Zhongdian Country, Yunnan	yyp08	2.72	3.95±0.93	5.96	3B	2*n*=8*x*=64=13m+37sm+14st(1sec)	
	Dulongjiang, Gongshan Country, Yunnan	yyp06	3.22	2.35±0.56	4.39	2B	2*n*=8*x*=64=1M+33m+27sm+3st	
	Wutoudi, Yulongxue Mountain, Lijiang, Yunnan						2*n*=8*x*=64=1M+33m+27sm+3st	
	Hong Mountain, Zhongdian Country, Yunnan						2*n*=8*x*=64=31m+15sm+15st+3t	Yang (2002)
	Bigusang, Zhongdian Country, Yunnan						2*n*=8*x*=64=38m+14sm+13st+1t	Yang (2002)
	Yuhu, Yulongxue Mountain, Lijiang, Yunnan						2*n*=8*x*=64	
	Baimaxue Mountain, Deqin Country, Yunnan						2*n*=8*x*=64	
	LBaimaxue Mountain, Deqin Country, Yunnan	yyp10					8*x*	
C. scaposa	Gao Mountain, Kangding Country, Sichuan	yyp02	3	2.65±0.75	4.64	2B	2*n*=4*x*=32=19m+13sm	
	Dege Country, Sichuan	yyp01	2.3	3.38±0.79	2.54	2B	2*n*=4*x*=32=1M+26m+5sm	
	Daofu Country, Sichuan	yyp03	2.82	2.03±0.42	2.42	2B	2*n*=4*x*=32=29m+2sm+1st	
	Shiqu Country, Sichuan						2*n*=4*x*=32=19m+11sm+2st	Yuan and Yang (2006)
	Hongyuan Country, Sichuan						2*n*=4*x*=32=13m+17sm+2st	Yang (2002)
	Xindu Bridge, Kangding Country, Sichuan						2*n*=4*x*=32=20m+8sm+4st	Yuan and Yang (2006)

	Chengduo Country, Qinghai						4*x*	
	A'ba Country, Sichuan	yyp05	3.19	3.71±1.02	4.91	2B	2*n*=8*x*=64=3M+45m+12sm+4st	
	Sejila Mountain, Linzhi Country, Tibet						2*n*=8*x*=64=30m+26sm+6st+2t	
	Xiaozhongdian, Zhongdian, Yunnan						2*n*=8*x*=64+2B	Yuan and Yang (2006)

注：LC：最长染色体长度；SC：最短染色体长度；CL：染色体平均长度。

Note: LC: longest chromosome length; SC: shortest chromosome length; CL: mean length of chromosome.

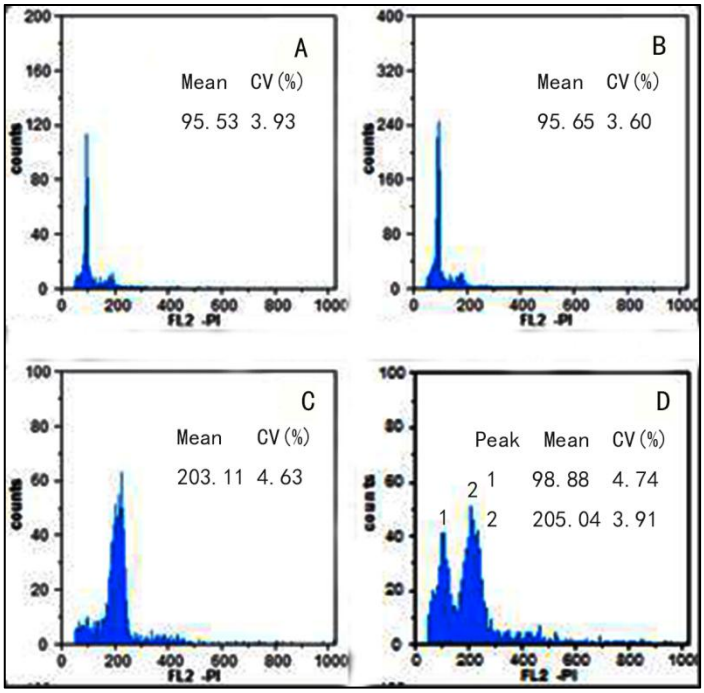


A. 驴蹄草（yyp04）； B. 驴蹄草（yyp06）； C. 驴蹄草（yyp07）； D. 驴蹄草（yyp08）；E. 花葶驴蹄草（yyp01）； F. 花葶驴蹄草（yyp02）； G. 花葶驴蹄草（yyp03）； H. 花葶驴蹄草（yyp05）。标尺 = 5 微米.

A. *C. palustris* (yyp04); B. *C. palustris* (yyp06); C. *C. palustris* (yyp07); D. *C. palustris* (yyp08); E. *C. scaposa* (yyp01); F. *C. scaposa* (yyp02); G. *C. scaposa* (yyp03); H. *C. scaposa* (yyp05). Scale bar = 5 μm.

图 1 驴蹄草和花葶驴蹄草的有丝分裂中期细胞图

Fig. 1 Mitotic nuclei and metaphase chromosomes of *C. palustris* and *C. scaposa*



A. 标样（yyp04）流式图；B. 样品（yyp 09）流式图；C. 样品（yyp 10）流式图；D. 样品（yyp 09 and yyp10）流式图。

A. The peak at the G0/G1 phase of standard (yyp04); B. The peak at the G0/G1 phase of sample (yyp 09); C. The The peak at the G0/G1 phase of sample (yyp 10); D. The peaks marked 1 and 2 at the G0/G1 phase of sample (yyp 09 and yyp10).

图 2 居群 yyp09 和 yyp10 流式图

Fig. 2 Flow cytometry (FCM) histograms of populations yyp09 and yyp 10

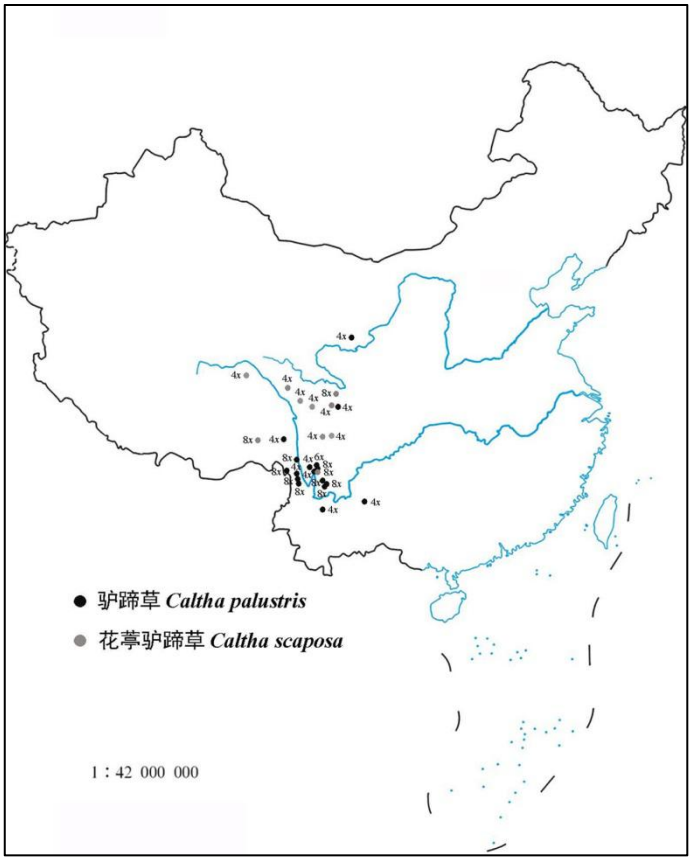


图 3 驴蹄草和花葶驴蹄草倍性分布图，黑色和灰色圆圈分别代表驴蹄草和花葶驴蹄草

Fig. 3 Distribution of cytotypes of *C. palustris* and *C. scaposa*, Black and gray circle indicated *C. palustris* and *C. scaposa*, separately